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**14. ABSTRACT**
Optimal early detection and prevention strategies for breast cancer are predicated on our ability to identify individuals at significantly increased risk for this disease. The purpose of this Center is to bring molecular risk prediction for breast cancer into the clinical area. This will require progress on three fronts of scientific endeavor:(i) Establishment of a tissue repository of benign breast disease; (ii) Assessment of potential biomarkers of risk in this tissue set and (iii) Discovery of new, potentially relevant biomarkers of risk. We have made significant progress on these aims. Our cohort is comprised of 9,376 women, 758 (8%) of whom have been diagnosed with breast cancer since the time of their benign biopsy. We established our tissue repository of benign breast tissue and have collected the subsequent breast cancer tissue. We assessed the significance of benign histology in predicting risk of future breast cancer, examining in detail the role of proliferative disease, atypia, papillomas, radial scars and involution. We explored the link between centrosome amplification, COX-2 expression and breast cancer outcomes and are currently exploring the significance of p16, ER and Ki67. We are working with Wayne State to characterize the histopathology in a cohort of African American women. Our focus in 2008-2009 will be on the Wayne State cohort.

**15. SUBJECT TERMS**
benign breast disease, biomarkers, histology, breast cancer

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**Benign Breast Disease: Toward Molecular Prediction of Breast Cancer Risk**

## INTRODUCTION

We are currently under a no cost extension period to complete the Wayne State cohort activities and biomarker analyses. This report provides our accomplishments in the past year.

## Task 1: Establish Retrospective Cohort of BBD and Nested Case-Control Study

A. Complete cohort follow-up
We reported the details in our 2006 report. This task has been completed.

B. Validate reported breast cancers
We reported the details in our 2006 report. This task has been completed.

C. Match appropriate controls to known breast cancer cases
We described this process in our 2004 report. This task has been completed.

D. Construct test set for preliminary evaluation of markers
We described the construction of our test set in our 2004 report. This subset consists of 124 cases and their two closest controls selected from the entire study period.

E. Construct validation set from remaining breast cancer cases, each matched with two controls.
The remaining cases and controls will serve as the validation set.

## Task 2: Biomarkers in Archived Tissues from Cases and Controls

A. Retrieve tissue slides/blocks of BBD specimens for all cases and controls
We reported details in our 2006 report. This task has been completed.

B. Characterize benign histopathology
1. General findings
In 2006 we reported the benign histology for our entire cohort. This objective has been completed. We published the general histology findings in July 2005 in the *New England Journal of Medicine.*

2. Atypia
We reported on our atypia results in our 2007 report. These results were published in the July 1, 2007 issue of *Journal of Clinical Oncology.*.

3. Papillomas
These data and the publication of these data were reported in 2006.

4. Involution
In our 2006 report we identified that the extent of lobular involution in breast tissue is an important risk indicator for the development of breast cancer. These results were published in the *Journal of the National Cancer Institute* in November, 2006.

5. Radial Scars

We reported the details in our 2007 report. These results were published on line in *Breast Cancer Research Treatment* (5/22/07) and in print 2008; 108:167-74.

C. Prepare tissue slides for biomarker analyses

Tissue slides have been prepared as various biomarkers have been explored.

D. Perform IHC of molecular markers

Our focus continues to be on the earliest possible changes that we might detect in these "premalignant" lesions. Our focus this past year has been on COX-2, ER alpha, Ki67 and p16.

1. COX-2 in atypia

We reported these findings in our 2006 and 2007 reports. These results were published in the March 19, 2008 *Journal of the National Cancer Institute*

> Visscher DW, Pankratz VS, Santisteban M, Reynolds C, Ristimäki A, Vierkant RA, Lingle WL, Frost MH, Hartmann LC. Association between cyclooxygenase-2 expression in atypical hyperplasia and risk of breast cancer. Journal of the National Cancer Institute 2008;100(6):421-7.

2. ER

We reported these findings in our 2007 report. These results are being prepared for publication.

3. Ki-67

Ki67 is a protein expressed in actively dividing cells, a feature associated with malignant cells and precancerous cells. We have stained all the atypias for Ki-67 and read them using the Automated Cellular Imaging System (ACIS). The question is whether or not proliferation status of benign breast tissue will predict subsequent development of breast cancer. Analyses of the date are underway.

4. p16

p16 is a very important regulatory element within cells. In a normal cell that is stressed, or beginning to grow out of control, p16 levels should rise and stop further cell growth. But at least some precancerous cells have the ability to grow through the p16 stop signal. Increased expression of p16 has been associated with a greater chance that women diagnosed with ductal carcinoma in-situ (DCIS) will experience a recurrence of their breast cancer after a lumpectomy. We are exploring whether p16 has any significance in identifying women with a benign breast biopsy who will go on to develop breast cancer. Our preliminary analyses do not show that this marker alone signals an increased risk of breast cancer. However, p16 increases as ER increases in staining intensity. Next, we will be exploring how p16 may be interacting with other biomarkers.

E. Perform centromere studies.

These data were presented in our 2006 report. Task completed.

**Task 3: Discovery - *In Vitro* Culturing and Gene Profiling Studies**

A. Identify appropriate BBD specimens for profiling.
The purpose of these studies is to identify new, potentially relevant biomarkers in benign breast disease, markers that might correlate with subsequent breast cancer risk. When our grant was submitted, the technology was not available to do profiling studies in paraffin-embedded tissue (such as our BBD resource). Fortunately, genomic profiling technology has proceeded significantly and there are now platforms available for us where microdissected, paraffin-embedded samples can be run. Because of the importance of lobular involution in reducing risk of breast cancer (see Task 2.B.4 above), and because there is no information currently on mediators of involution, we have selected paraffin-embedded samples that display varying degrees of involution for profiling. First we have performed pilot studies on test formalin-fixed paraffin embedded samples. For these trial studies, we have been using paraffin-embedded tissue from transgenic mice that express a matrix metalloproteinase (MMP) that is expressed during postlactational involution. We identified a method for preparing RNA of sufficient quality from paraffin-blocked tissue, and then worked with the Mayo Clinic Microarray Core to optimize the microarray processing protocols. Through this approach, we have identified a procedure that can be used with the cohort biopsies (Figure 2).

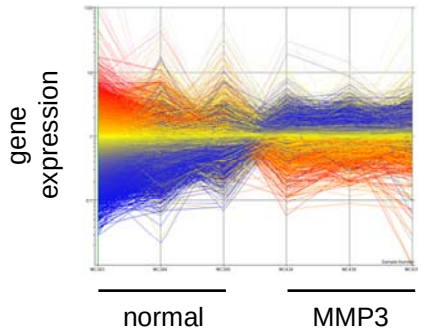


**Figure 2. Transcriptional profiling of formalin fixed, paraffin-embedded tissue.** The graph represents expression levels of 45,000 genes as individual lines; each line connects the gene expression levels in three nontransgenic normal mice (left) and three transgenic MMP3 mice (right). The lines are colored according to expression levels in the normal mice; genes with high expression in the normal mice are colored red, and genes with low expression in the normal mice are colored blue.

B. Obtain fresh BBD tissue from appropriate patients at Mayo for culturing in vitro at UCSF.
Forty-four samples were sent from Mayo to UCSF. Five of these samples were lost to contamination. Task completed.

C. Culture BBD specimens and document their growth characteristics.
These data were reported in 2005. Task completed.

D. Compare genomic expression levels of DCIS markers in BBD tissues.
We reported on this task in 2006. Task completed.

E. Profile BBD specimens.
We reported on this task in 2006. Task completed.

**Task 4: Statistical Analyses**

A. Establishment of relational database
This task is complete. The database is the foundation for tracking all tissue samples; entering clinical, pathologic, and molecular data; and analyzing results.

B. Enter epidemiologic and histopathologic data
This task is complete.

C. Enter culturing data (proportion of cells that break through proliferation barriers; slope of curve, etc.)
These data were entered as collected at UCSF.

D. Enter molecular data from culturing experiments (methylation of p16, p53 status, % proliferation versus apoptosis, etc).
These data were entered as collected at UCSF.

E. Enter gene profiling data.
These data were entered as collected at UCSF.
.
F. Calculate hazard function for breast cancer by age at BBD, family history, histology, and molecular marker data.
We reported these findings in the 2007 report.

G. Assess accuracy of Gail model.
We reported these findings in the 2007 report. The manuscript has been accepted for publication in the *Journal of Clinical Oncology*.

Pankratz VS, Hartmann LC, Degnim AC, Vierkant RA, Ghosh K, Vachon CM, Frost MH, Maloney SD, Reynolds C, Boughey JC. Assessment of the accuracy of the Gail Model in women with atypical hyperplasia. *Journal of Clinical Oncology*. Accepted for publication.

G. Analyze expression data.
This past year we have focused on ER, p16, Ki-67. We have reported our current status under Task 2.

**Task 5: Compare Breast Cancer Risk Associated with Benign Breast Disease in African-American vs. Caucasian-American Women**

A. Identify African-American women at Wayne State University who had a breast biopsy with benign results between 1992 and 2001.
Through a collaboration with Dr. Hind Nassar, an IRB-approved protocol has been developed, to access paraffin-embedded samples of benign breast disease (BBD) from African-American women at Wayne State from 1992-2001. This will allow us to begin to look at the problem of BBD in African-American women. Moreover, because the population there is covered through the Detroit SEER database, we will have information about cancer outcomes. During this year our investigator from Wayne State, Dr. Hind Nassar, has moved to Johns Hopkins University. She has been given the authorization by both Hopkins and Wayne State to complete this work. She will return to Wayne State to complete the readings on a monthly basis. Dr. Nassar has received IRB approval at Johns Hopkins and Wayne State to complete this project.

B. Retrieve slides/blocks of BBD specimens.
Slides and blocks continue to be retrieved as Dr. Nassar is able to complete readings.

C. Characterize benign histology of epithelium.
Dr. Nassar continues to read and enter the histology into the data entry tool developed by the Mayo programmer.

D. Cross list with Detroit SEER database to identify subsequent breast cancers.

Dependent on A-C.

E. Data clean-up, compare age, histology, involution status, and resulting risk with Mayo Caucasian-American cohort and determine involution status by age of patient.
To begin once A – D accomplished.

**KEY RESEARCH ACCOMPLISHMENTS**

- We identified the degree of risk associated with the common benign epithelial entities and the extent to which age at biopsy and family history influence the risk of breast cancer in women with proliferative or atypical lesions. The highest risk was among women who had proliferative disease with atypia, especially those of younger age.

- We identified a marked increased risk of breast cancer in women with three or more foci of atypia, especially for three or more foci with calcifications. Also, risk was higher in women diagnosed with atypical hyperplasia before age 45. Among women with atypia, risk was not affected by family history.

- We identified that a single papilloma without atypia imparts an increased risk of developing a subsequent carcinoma similar to other forms of proliferative breast disease without atypia. Atypical papilloma, particularly in the setting of multiple papillomas, imparts a breast cancer risk similar to or greater than conventional atypical ductal/lobular hyperplasias.

- We identified that the extent of lobular involution in breast tissue is an important risk indicator for the development of breast cancer. Increasing degrees of involution result in a significant reduction in breast cancer risk, even in women at “high risk” based on atypia or young age.

- We found that intense COX-2 expression is associated with a significantly greater likelihood of a subsequent breast cancer in women with atypia and represents one potential molecular target for chemoprevention strategies.

- We found no increased breast cancer risk for women with radial scars compared to the risk already present due to proliferative disease with or without atypia.

- We identified that centrosome amplification is seen more frequently in higher risk benign lesions (e.g. atypia) and is infrequently seen in non-proliferative lesions and in proliferative lesions without atypia.

- Our preliminary data show that p16 alone does not signal an increased risk of breast cancer. However, p16 increases as ER increases in staining intensity.

- We found the Gail model to predict no better than chance alone the breast cancer risk of women with atypia. The model significantly underestimated lifetime risk of our cohort of women with atypia.

**REPORTABLE OUTCOMES**
**Manuscripts**

- Degnim AC, Visscher DW, Berman HK, Frost MH, Sellers TA, Vierkant RA, Maloney SD, Pankratz VS, deGroen PC, Lingle WL, Ghosh K, Penheiter L, Tlsty T, Melton LJ, Reynolds CA, Hartmann LC. Stratification of breast cancer risk in women with atypia: A Mayo cohort study, *Journal of Clinical Oncology* 2007;25(19):2671-7.
- Berg JC, Visscher DW, Vierkant RA, Pankratz VS, Maloney SD, Lewis JT, Frost MH, Ghosh K, Degnim AC, Brandt KR, Vachon CM, Reynolds CR, Hartmann LC. Breast cancer risk in women with radial scars in benign breast biopsies. *Breast cancer Research and Treatment* 2008;108:167-174.
- Visscher DW. Pankratz VS. Santisteban M. Reynolds C. Ristimaki A. Vierkant RA. Lingle WL. Frost MH. Hartmann LC. Association between cyclooxygenase-2 expression in atypical hyperplasia and risk of breast cancer. *Journal of the National Cancer Institute* 2008;100(6):421-7.
- Pankratz VS, Hartmann LC, Degnim AC, Vierkant RA, Ghosh K, Vachon CM, Frost MH, Maloney SD, Reynolds C, Boughey JC. Assessment of the accuracy of the Gail Model in women with atypical hyperplasia. *Journal of Clinical Oncology*. Accepted for publication.

**Presentations**

**Poster Presentation at American Society of Clinical Oncology's Breast Cancer Symposium, San Francisco, CA, September 2007.**

- Boughey JC, Hartmann LC, Degnim AC, Vierkant RA, Ghosh K, Vachon CM, Maloney SD, Reynolds C, Pankratz VS. Assessment of the accuracy of the Gail model in women with atypical hyperplasia.

**CONCLUSIONS**

This past year we have focused on biomarker data and the characterization of benign breast disease in the Wayne State African-American cohort. We have made significant progress on our biomarker data. We have published results on our COX-2 data, an article was accepted on our Gail model data and we are preparing ER and p16 data for publication. We are currently analyzing Ki-67 data. Work on the Wayne State African-American cohort was slowed by the fact that our Wayne State collaborator, Dr. Hind Nassar, moved from Wayne State to Johns Hopkins this past year. We have worked with Dr. Nassar to accomplish the logistics that will make it possible for her to return periodically to Wayne State to complete this work. Our main focus this upcoming grant year will be completion of the Wayne State cohort and data analyses comparing our Caucasian-American cohort with the African-American cohort.

Appendix A: COX-2 article

ARTICLE

# Association Between Cyclooxygenase-2 Expression in Atypical Hyperplasia and Risk of Breast Cancer

Daniel W. Visscher, V. Shane Pankratz, Marta Santisteban, Carol Reynolds, Ari Ristimäki, Robert A. Vierkant, Wilma L. Lingle, Marlene H. Frost, Lynn C. Hartmann

**Background** The cyclooxygenase-2 (COX-2) enzyme, which is induced by inflammatory and mitogenic stimuli, plays a protumorigenic role in several human cancers. COX-2 is overexpressed in invasive and in situ breast cancers. Atypical hyperplasia in breast tissue, although benign, is associated with a high risk of breast cancer. We investigated whether COX-2 overexpression in atypical hyperplasia is associated with the risk of subsequent breast cancer.

**Methods** COX-2 expression was assessed immunohistochemically in archival sections from 235 women with atypia whose biopsy specimens were obtained at the Mayo Clinic from January 1, 1967, through December 31, 1991. COX-2 expression was scored as 0 (negative), 1+ (weak), 2+ (moderate), or 3+ (strong). Risk factor information and follow-up for breast cancer events were obtained via a study questionnaire and the medical records. All statistical tests were two-sided.

**Results** Forty-one (17%) of the 235 women developed breast cancer during a median follow-up of 15 years. Moderate (category 2+) or strong (category 3+) COX-2 expression was identified in 71 (30%) and 34 (14%) of the 235 samples, respectively. The risk for developing breast cancer, relative to a control population (the Iowa Surveillance, Epidemiology, and End Results registry), increased with increasing COX-2 expression (relative risk [RR] = 2.63, 95% confidence interval [CI] = 1.56 to 4.15, for those with negative or weak COX-2 expression; RR = 3.56, 95% CI = 1.94 to 5.97, for those with moderate expression; and RR = 5.66, 95% CI = 2.59 to 10.75, for those with strong expression; $P = .07$). Overexpression of COX-2 was statistically significantly associated with the type of atypia (lobular vs ductal, $P < .001$), number of foci of atypia in the biopsy ($P = .02$), and older age at time of biopsy (>45 years, $P = .01$).

**Conclusions** COX-2 appears to be a biomarker that further stratifies breast cancer risk among women with atypia and may be a relevant target for chemoprevention strategies.

J Natl Cancer Inst 2008;100:421–427

Women with atypical hyperplasia are at high risk for the development of breast cancer, with a cumulative incidence approaching 25% at 20 years after biopsy examination (1,2). Clinical practice would benefit from the identification of additional predictive features for women with atypia that will enable better risk stratification for these women and from the identification of functional biologic targets that could be modified by chemoprevention strategies.

Cyclooxygenase (COX) enzymes catalyze the synthesis of bioactive prostaglandins from arachidonic acid, which is derived from membrane phospholipids (3). Cyclooxygenase-2 (COX-2), induced in response to various inflammatory and mitogenic stimuli, has been shown to play a protumorigenic role in preclinical models of several tumor systems (4–6). Moreover, it is overexpressed in several human cancers and their precancerous lesions (7). Ristimäki et al. (8) analyzed COX-2 expression by immunohistochemistry in 1576 invasive breast cancer specimens and found moderate to strong expression in 37% of the cancers. In ductal carcinoma in situ, the frequency of COX-2 overexpression appears to be even higher (9,10). Many groups have shown an association between COX-2 expression and an aggressive phenotype in in situ and invasive breast cancer (8–15). In animal models, COX inhibitors suppress experimental breast cancers (6,7), and several epidemiologic studies in humans have shown that nonsteroidal anti-inflammatory drug (NSAID) use is associated with a reduced incidence of breast cancer (3,16,17).

**Affiliations of authors:** Department of Surgical Pathology, University of Michigan, Ann Arbor, MI (DWV); Divisions of Biostatistics (VSP, RAV), Medical Oncology (MS, MHF, LCH), Anatomic Pathology (CR), and Experimental Pathology (WLL), Mayo Clinic, Rochester, MN; Department of Pathology, HUSLAB/Helsinki University Central Hospital and Genome-Scale Biology Research Program, Biomedicum Helsinki, University of Helsinki, Helsinki, Finland (AR).

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See "Funding" and "Notes" following "References."

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## CONTEXT AND CAVEATS

**Prior knowledge**

Cyclooxygenase-2 (COX-2) plays a protumorigenic role in several human cancers and is overexpressed in invasive and in situ breast cancers. Atypical hyperplasia in breast tissue, although benign, is associated with a high risk of breast cancer.

**Study design**

The relationship between the risk of breast cancer and COX-2 expression in archival specimens from women with atypical hyperplasia and a 15-year follow-up was assessed.

**Contribution**

The risk for developing breast cancer, relative to a control population (the Iowa Surveillance, Epidemiology, and End Results registry), increased with increasing COX-2 expression, in a borderline statistically significant manner. Overexpression of COX-2 was statistically significantly associated with the type of atypia (lobular vs ductal), with number of foci of atypia in the biopsy, and with older age at time of biopsy (≥45 years).

**Implications**

COX-2 may be a biomarker that further stratifies breast cancer risk among women with atypia and may be a relevant target for chemoprevention strategies.

**Limitations**

Tissue-based biomarker studies, such as this study, are limited by semiquantitative and subjective evaluation of COX-2 status, which is further complicated by the variable nature of immunoreactivity.

Because of the relevance of COX-2 to breast cancer biology, including its presence in preinvasive lesions, we hypothesized that COX-2 expression is increased in atypia and that overexpression is a risk factor for the progression of breast cancer. Accordingly, we studied COX-2 expression in a cohort of women with atypical hyperplasia for whom we have long-term cancer follow-up information.

## Participants and Methods

### Study Population

Entry criteria for the study cohort have been described previously (1,2). Briefly, this study included all women aged 18–85 years who had a benign breast biopsy that was surgically excised at the Mayo Clinic from January 1, 1967, through December 31, 1991. The initial cohort included 9087 women (1). With additional follow-up, data for 9376 women were available for this analysis, 331 (3.5%) of whom had atypical hyperplasia (2). Archival paraffin-embedded, formalin-fixed tissue for COX-2 staining was available for 235 of the 331 women.

### Risk Factor Information and Follow-up

Follow-up for breast cancer events and risk factor information were obtained for all 235 women with atypia through Mayo medical records and a study questionnaire (1,2). Family history was collected via respondent questionnaires and medical record abstraction and classified as negative, strong, or weak. Criteria for a strong family history were at least one first-degree relative with breast cancer diagnosed before the age of 50 years or two or more relatives with breast cancer, with at least one being a first-degree relative. Any lesser degree of family history was considered to be weak.

All protocol procedures and patient contact materials were reviewed and approved by the Institutional Review Board of the Mayo Clinic. Return and completion of the patient contact materials were considered to be implied consent.

### Histology

All archival hematoxylin- and eosin-stained sections were evaluated by the study breast pathologist (DWV), without knowledge of the original histologic diagnosis or patient outcome. A diagnosis of atypical ductal hyperplasia or atypical lobular hyperplasia was based on the criteria of Page et al. (18) and Page and Rogers (19). For each example of atypical hyperplasia, the number of separate foci was defined (2). Multifocal atypia required the identification of atypia in more than one terminal duct lobular unit, as defined by clear separation from another terminal duct lobular unit by nonspecialized interlobular mammary stroma.

### Immunostaining

Five-micrometer sections of formalin-fixed, paraffin-embedded samples were deparaffinized with three changes of xylene, rehydrated in an ethanol series (100%, 95%, and 70% ethanol), and rinsed well in running distilled water. Slides were then placed in preheated epitope retrieval buffer (1 mM EDTA, pH 8.0) for 30 minutes, cooled in the buffer for 5 minutes, and rinsed for 5 minutes in running distilled water. An automated slide stainer (AS100 Autostainer Plus, DAKO, Carpinteria, CA) was used for all slides at room temperature as follows. Sections were first incubated in a solution of 3% $H_2O_2$ in ethanol for 5 minutes to inactivate endogenous peroxides and then incubated in primary mouse anti–human COX-2, Clone CX-294, monoclonal antibody (1:100 dilution; M3617, DAKO) for 30 minutes. Sections were rinsed with TBST 10x wash buffer (S3006, tris-buffered saline with Tween 20, DAKO). Sections were then incubated with a peroxidase-labeled polymer conjugated to goat anti-mouse, anti-rabbit immunoglobulins (EnVision+ Dual Link System-HRP, K4061, DAKO) for 15 minutes. The slides were rinsed with TBST wash buffer, incubated in Nova Red (Vector Laboratories, Burlingame, CA) for 5 minutes, and counterstained with modified Schmidts' hematoxylin for 5 minutes and rinsed for 3 minutes in tap water to set the hematoxylin counterstain. Specimens were dehydrated through a graded ethanol series (70%, 95%, and 100% ethanol), cleared in three changes of xylene, and mounted with a permanent mounting medium. The positive control was colon cancer tissue; the negative control was normal colonic epithelial tissue. All samples were stained simultaneously over a 2-day period.

### Evaluation of COX-2 Immunostaining

COX-2 immunostaining was analyzed by the study breast pathologist (DWV), who had no knowledge of patient outcome. The following criteria were established before the reading of the samples (by AR and DWV) (8): 0 = no staining; 1+ = weak, barely perceptible staining in a patchy cytoplasmic pattern; 2+ = moderate intensity staining, usually cytoplasmic, with focal plasma membrane distribution; 3+ = strong immunoreactivity with distinct plasma

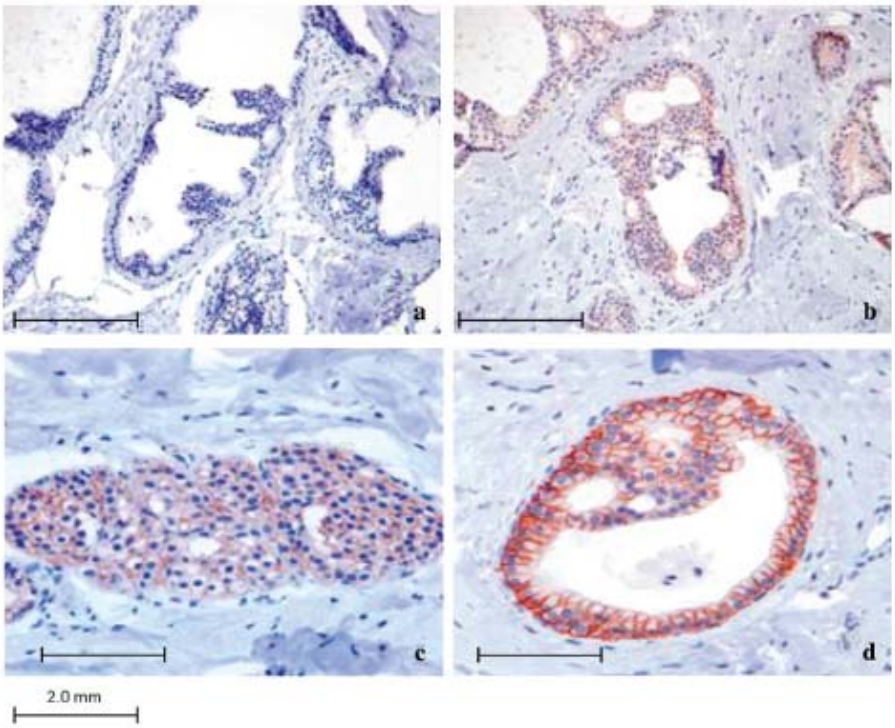


**Figure 1.** Cyclooxygenase-2 staining patterns in atypia. Representative breast atypia samples are shown. Other samples with corresponding scores were similar. a) Category 0 (no) staining. b) Category 1+ staining. c) Category 2+ staining. d) Category 3+ staining. Original magnification was ×200. Scale bar = 2.0 mm.

membrane accentuation (Figure 1). Because of the small number of breast cancer events in samples with 0 staining, categories 0 and 1+ were combined for all analyses.

### Statistical Analyses

Data were summarized descriptively by use of frequencies and percentages for categorical variables and medians and interquartile ranges (IQRs) for continuous variables. We compared distributions of demographic and clinical attributes from patients with and without available formalin-fixed tissue (for all 331 women with atypia) and across all levels of staining intensity (for the 235 women with COX-2 immunostaining results) by use of $\chi^2$ tests.

The length of follow-up for each woman in the study was calculated as the number of days from her benign biopsy to the date of her breast cancer diagnosis, death, or last contact. We estimated relative risks (RRs) on the basis of standardized incidence ratios, by dividing the observed numbers of incident breast cancers by population-based expected values. This approach allowed us to compare rates of breast cancer in our cohort with that of the general population rather than an internal referent group, recognizing that all women in our cohort were at some increased risk of breast cancer from their diagnosis of atypical hyperplasia. Expected values were calculated by apportioning each woman's person-years of follow-up into 5-year age and calendar-period categories and multiplying these by the corresponding breast cancer incidence rates from the Iowa Surveillance, Epidemiology, and End Results registry (1). This reference population was chosen because of its demographic similarities to the Mayo Clinic population (80% of cohort members reside in the upper Midwest). Potential heterogeneity in standardized incidence ratios across levels of COX-2 staining was assessed by use of Poisson regression analysis, with the log-transformed expected event rate for each individual modeled as the offset term. We displayed observed event rates by use of cumulative incidence curves, accounting for the effects of death as a competing risk (20). We assessed whether COX-2 staining intensity was associated in a dose–response manner with cumulative incidence by use of tests for trend, which were calculated via Cox proportional hazards regression analysis. We tested for departures from the proportional hazards assumption by use of tests of interaction with follow-up time and found no evidence of nonproportionality. Among breast cancer patients, we compared time to cancer across levels of immunostaining with analysis of variance (ANOVA) methods and laterality of breast cancer relative to the original atypia across levels of immunostaining by use of $\chi^2$ tests. All statistical tests were two-sided, and all analyses were conducted with the SAS (SAS Institute, Inc., Cary, NC) software system.

## Results

### COX-2 Immunostaining of Atypia Samples

Among the original cohort of 331 women with atypical hyperplasia (2), the distributions of breast cancer status, age at benign biopsy, family history of cancer, and (for breast cancer patients) time to diagnosis were not statistically significantly different between the 235 patients with formalin-fixed tissue available for COX-2 staining and the 96 patients without available tissue ($\chi^2$ $P > .05$ for each attribute). Among specimens from the 235 patients with available tissue, 23 (10%) showed no COX-2 staining, 107 (46%) showed category 1+ staining, 71 (30%) showed category 2+ staining, and 34 (14%) showed category 3+ staining (Figure 1). Intensity of staining was statistically significantly associated with type of atypical hyperplasia (lobular vs ductal, $P < .001$; Table 1). Of the 100 women with atypical ductal hyperplasia only, 77 (or 77%) had either no or weak (categories 0 or 1+) COX-2 staining, 13 (13%) had moderate (category 2+) staining, and 10 (10%) had strong (category 3+) staining.

**Table 1.** Association of cyclooxygenase-2 staining intensity with demographic and clinical variables, among women diagnosed with atypical hyperplasia*

| Variable | COX-2 staining category | | | *P* value† |
|---|---|---|---|---|
| | 0–1+ (n = 130) | 2+ (n = 71) | 3+ (n = 34) | |
| Age at benign biopsy, No. (%) | | | | .01 |
| <45 y | 24 (80.0) | 6 (20.0) | 0 (0.0) | |
| 45–55 y | 44 (58.7) | 21 (28.0) | 10 (13.3) | |
| >55 y | 62 (47.7) | 44 (33.8) | 24 (18.5) | |
| Family history of breast cancer‡, No. (%) | | | | .95 |
| None | 74 (54.4) | 41 (30.1) | 21 (15.4) | |
| Weak | 22 (53.7) | 14 (34.1) | 5 (12.2) | |
| Strong | 26 (57.8) | 12 (26.7) | 7 (15.6) | |
| No. of atypical foci, No. (%) | | | | .02 |
| 1 | 84 (64.1) | 31 (23.7) | 16 (12.2) | |
| 2 | 31 (47.7) | 25 (38.5) | 9 (13.8) | |
| ≥3 | 15 (38.5) | 15 (38.5) | 9 (23.1) | |
| Calcifications, No. (%) | | | | .97 |
| Yes | 40 (54.1) | 23 (31.1) | 11 (14.9) | |
| No | 90 (55.9) | 48 (29.8) | 23 (14.3) | |
| Involution status§, No. (%) | | | | .27 |
| None | 9 (64.3) | 3 (21.4) | 2 (14.3) | |
| Partial | 104 (58.1) | 51 (28.5) | 24 (13.4) | |
| Complete | 14 (38.9) | 14 (38.9) | 8 (22.2) | |
| Type of atypical hyperplasia, No. (%) | | | | <.001 |
| ALH | 47 (38.5) | 53 (43.4) | 22 (18.0) | |
| ADH | 77 (77.0) | 13 (13.0) | 10 (10.0) | |
| ALH and ADH | 6 (46.2) | 5 (38.5) | 2 (15.4) | |
| Indication for biopsy‖, No. (%) | | | | .14 |
| Lump | 48 (50.5) | 36 (37.9) | 11 (11.6) | |
| Mammogram | 78 (57.8) | 35 (25.9) | 22 (16.3) | |
| Vital status, No. (%) | | | | .09 |
| Deceased | 38 (52.1) | 19 (26.0) | 16 (21.9) | |
| Alive | 92 (56.8) | 52 (32.1) | 18 (11.1) | |
| Year of biopsy, No. (%) | | | | .22 |
| 1967–1971 | 6 (66.7) | 2 (22.2) | 1 (11.1) | |
| 1972–1976 | 12 (48.0) | 12 (48.0) | 1 (4.0) | |
| 1977–1981 | 15 (51.7) | 7 (24.1) | 7 (24.1) | |
| 1982–1986 | 33 (49.3) | 21 (31.3) | 13 (19.4) | |
| 1987–1991 | 64 (61.0) | 29 (27.6) | 12 (11.4) | |

* COX-2 = cyclooxygenase-2; ALH = atypical lobular hyperplasia; ADH = atypical ductal hyperplasia. Values expressed as number (percent).

† $\chi^2$ test of statistical significance.

‡ Family history was available for 222 of the 235 women. Criteria for a strong family history were at least one first-degree relative with breast cancer diagnosed before the age of 50 years or two or more relatives with breast cancer, with at least one being a first-degree relative. Any lesser degree of family history was considered to be weak.

§ Involution status was available for 229 of the 235 women.

‖ Indication information was available for 230 of the 235 women.

In contrast, of the 122 women with only atypical lobular hyperplasia, 47 (39%) had no or weak staining, whereas 53 (43%) had moderate staining and 22 (18%) had strong staining ($P < .001$). Strong immunostaining was also more likely with increasing patient age: no tumor from the 30 women who were younger than 45 years at time of initial biopsy had strong COX-2 staining and only six (20%) had moderate staining. In contrast, among tumors from the 130 women who were older than 55 years at biopsy, 24 (19%) had strong staining and an additional 44 (34%) had moderate COX-2 staining ($P = .01$). Finally, the strength of COX-2 immunostaining was associated with increased numbers of atypia foci. Among the 39 patients with three or more foci, nine (23%) showed strong COX-2 staining and 15 (39%) showed moderate staining. In contrast, of the 131 women who had only a single focus of atypia, 16 (12%) had strong COX-2 staining and 31 (24%) had moderate staining ($P = .02$). The degree of COX-2 immunoreactivity was not associated with family history of breast cancer, calendar year of biopsy, or clinical indication for biopsy (palpable lump vs mammographic abnormality).

COX-2 staining was not limited to atypical foci. Of the 235 subjects in our cohort, 216 (92%) had staining detected in benign lobules, but it was heterogeneous within tissue sections and usually weak or moderate in intensity (category 0, 1+, or 2+ in 202 [94%] of the 216 patients). A total of 179 patients also had staining in (nonatypical) proliferative lesions, such as usual-type duct hyperplasia and adenosis that were present in the tissue sections along with atypia. Most of these lesions were also weakly immunoreactive (category 0 or 1+ in 131 [73%], category 2+ in 43 [24%], and category 3+ in five [3%] of the lesions).

### Association of COX-2 Expression With Breast Cancer Risk

Forty-one (17%) of the 235 women with atypia in this study developed breast cancer during a median follow-up of 15 years. Figure 2 illustrates the cumulative incidence of breast cancer as a function of follow-up interval, stratified by category of COX-2 staining intensity. A positive association of borderline statistical significance was observed between COX-2 staining and the subsequent development of breast cancer (test for trend $P = .07$, Cox proportional hazards regression). Risks of developing breast cancer after 15 years of follow-up among women with atypia were as follows: for atypia with no or only weak COX-2 staining, 13% (95% confidence interval [CI] = 6% to 20%); with moderate staining, 19% (95% CI = 7% to 30%); and with strong staining, 25% (95% CI = 6% to 43%). After a follow-up of 20 years, risks of developing breast cancer for atypia with category 0 or 1+, category 2+, or category 3+ staining were 14% (95% CI = 7% to 22%), 24% (95% CI = 10% to 37%), and 31% (95% CI = 8% to 53%), respectively. Poisson regression analyses that compared the observed number of events with the expected number and accounted for age and calendar period also revealed a marginally statistically significant dose–response relationship overall between staining intensity and risk of breast cancer, compared with that of the control population (for category 0 or 1+ COX-2 staining, RR = 2.63, 95% CI = 1.56 to 4.15; for category 2+ staining, RR = 3.56, 95% CI = 1.94 to 5.97; and for category 3+ staining, RR = 5.66, 95% CI = 2.59 to 10.75) (Table 2, test for heterogeneity of relative risks $P = .07$). Results were similar, although slightly attenuated, after further adjustment for number of atypical foci and type of atypia (atypical lobular hyperplasia or atypical ductal hyperplasia) (data not shown).

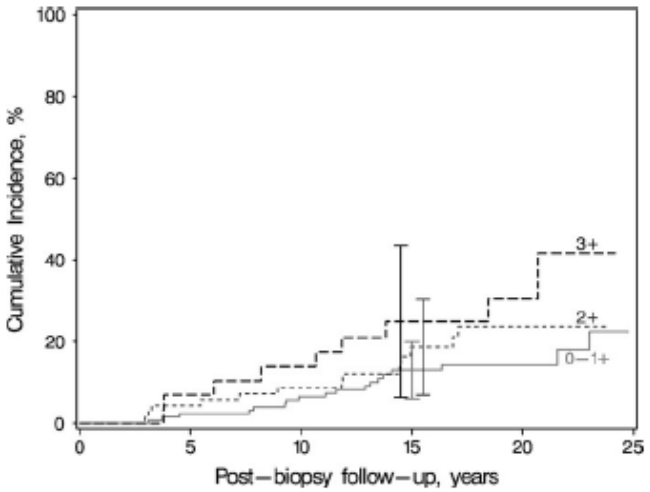


**Figure 2.** Cumulative breast cancer incidence among women with atypical hyperplasia, accounting for death as a competing risk. Data were stratified by level of cyclooxygenase-2 staining intensity (categories 0–1+, 2+, and 3+). Ninety-five percent confidence intervals about the cumulative incidence estimates at year 15 of follow-up are provided for reference. The number of subjects at risk by year of follow-up and staining intensity levels are as follows: baseline, 0–1+, n = 130; baseline, 2+, n = 71; baseline, 3+, n = 34; year 15, 0–1+, n = 63; year 15, 2+, n = 30; year 15, 3+, n = 11. Test for trend in risk across levels of intensity was used ($P_{trend} = .07$, Cox proportional hazards regression analysis).

### COX-2 Staining and Cancer Features

Among the 41 women who developed breast cancer during follow-up, 32 had invasive disease, eight had in situ cancer, and one had disease of an unknown type. We compared COX-2 staining intensity (categories 0 and 1+ vs categories 2+ and 3+) in the affected women by cancer type (invasive vs in situ) and found no difference (14 of the 18 women [78%] with category 0 or 1+ staining had invasive disease, as did 18 of the 22 [82%] with category 2+ or 3+ staining, $\chi^2$ $P = .75$).

We next examined the time to breast cancer by COX-2 staining intensity. Among the 41 women who developed breast cancer, the median time to breast cancer was 11.6 years (IQR = 7.2–14.5 years). Among the 18 women who developed breast cancer in the category 0 or 1+ COX-2 staining group, the median time to breast cancer was 11.4 years (IQR = 7.9–13.7 years); among the 23 who developed cancer in the category 2+ or 3+ staining group, the median time to breast cancer was 11.8 years (IQR = 5.5–15.0 years) (ANOVA $P = .87$). Side of breast cancer and side of atypia were known for 34 of the 41 women (14 in the category 0 or 1+ staining group and 20 in the category 2+ or 3+ group). In the category 0 or 1+ group, sidedness was equally distributed (seven ipsilateral and seven contralateral breast cancers). In the category 2+ or 3+ group, there were 13 (65%) ipsilateral and seven (35%) contralateral breast cancers; although seemingly different, these proportions were not statistically significantly different from each other ($\chi^2$ $P = .38$).

## Discussion

We studied COX-2 expression in a well-characterized cohort of women with atypical hyperplasia who were followed for breast cancer events for a median of 15 years. Among the atypias from women in this cohort, 44% had moderate or strong COX-2 expression,

**Table 2.** Association of cyclooxygenase-2 staining intensity with risk of breast cancer after the diagnosis of atypical hyperplasia*

| COX-2 staining intensity | No. of women | No. of person-years | No. of observed events | No. of expected events† | RR (95% CI)‡ |
|---|---|---|---|---|---|
| All women | 235 | 3265 | 41 | 12.4 | 3.31 (2.38 to 4.49) |
| Staining category | | | | | |
| 0–1+ | 130 | 1869 | 18 | 6.9 | 2.63 (1.56 to 4.15) |
| 2+ | 71 | 1004 | 14 | 3.9 | 3.56 (1.94 to 5.97) |
| 3+ | 34 | 391 | 9 | 1.6 | 5.66 (2.59 to 10.75) |

* COX-2 = cyclooxygenase-2; RR = relative risk; CI = confidence interval.

† Number of events expected on the basis of Iowa Surveillance, Epidemiology, and End Results data.

‡ Standardized incidence ratio and 95% confidence intervals, comparing observed number of breast cancers with those expected. All results account for age and calendar period.

and there was a borderline statistically significant increase in risk of later breast cancer associated with increasing levels of COX-2 expression ($P$ = .07). Atypias with more than one involved focus, which have the highest likelihood of progression to a later breast cancer (2), were more likely than those with just one involved focus to express COX-2.

There is strong biologic rationale underlying COX-2 as a relevant biomarker in breast carcinogenesis. COX-2, which is induced by mitogenic and inflammatory stimuli, has many protumorigenic downstream effects, including enhanced proliferation, enhanced angiogenesis, resistance to apoptotic cell death, immunosuppression, promotion of invasion, and metastasis (3–7,11,12). COX-2 is overexpressed in both invasive breast cancer and ductal carcinoma in situ, and overexpression is associated with aggressive histologic and clinical features (8–15). COX-2 is also overexpressed in preinvasive lesions in multiple tumor systems, including Barrett esophagus, colorectal adenomas, and cervical intraepithelial neoplasia (3,6,7).

We identified a relationship between older age at diagnosis of atypia and COX-2 overexpression. Specifically, only 20% of women who were younger than 45 years at diagnosis had atypias with moderate or strong COX-2 expression compared with 41% of women aged 45–55 years and 52.3% of women older than 55 years. Interestingly, these age-dependent differences in COX-2 induction could result in age-related changes in aromatase expression. The enzyme aromatase, encoded by the *CYP19* gene, synthesizes estradiol from androgenic precursors (7). Aromatase is present in breast tissue, and its levels are higher in or near breast cancers (21,22). It has been shown (23,24) that prostaglandin $E_2$, which is produced by enzymes downstream of COX-2, stimulates transcription of the *CYP19* gene. Thus, increased COX-2 expression and the resultant increased mammary aromatase activity would be expected to increase local estrogen concentrations, in turn further contributing to a protumorigenic local environment (7). This mechanism may be of greater importance in postmenopausal breast cancer, where the synthesis of estrogens is dependent on aromatase in peripheral tissues, especially mammary adipose tissue (25).

We found that elevated COX-2 expression was more common in atypical lobular hyperplasia than atypical ductal hyperplasia. Perrone et al. (26) also showed recently that lobular neoplasia expressed COX-2 at high levels. Lobular neoplasia is characterized by loss of adhesion molecules such as E-cadherin, and recent work (27,28) has shown that COX-2 and prostaglandin $E_2$ induce Snail, a transcriptional repressor that silences the E-cadherin gene *CDH1*. Our data in atypia suggest that COX-2 may be responsible, at least in part, for the protumorigenic loss of adhesion molecules that occurs in lobular neoplasia of the breast.

Many studies [for review, see Harris et al. (29)] have explored whether administration of NSAIDs, which inhibit COX-2, is associated with the subsequent risk of breast cancer. Recent results from a case–control study (17) show that users of selective COX-2 inhibitors and nonselective inhibitors, including regular aspirin, ibuprofen, or naproxen, had a reduced risk of subsequently developing breast cancer, whereas users of acetaminophen did not. A previous meta-analysis (16) found a link between regular use of NSAIDs and reduction in breast cancer risk. These data supplement the extensive literature that supports the use of selective COX-2 inhibitors as chemoprevention agents for other cancers, most notably gastrointestinal malignancies.

This study has several limitations. Tissue-based biomarker studies, such as this study, are necessarily limited by semiquantitative and subjective evaluation of COX-2 status, which is further complicated by the variable and generally weak character of immunoreactivity. More quantitative approaches, such as quantitative reverse transcriptase–polymerase chain reaction or western blotting, typically require fresh frozen tissue and cannot accurately localize the COX-2 signal in the tissue because of the small size of the atypical foci. The advantages of immunohistochemistry are that we can localize the COX-2 signal to the atypia foci and that we can use archival paraffin-embedded material from patients with adequate follow-up to determine later breast cancer events. One member of our investigative team (AR) has compared various antibodies and approaches in cancer samples in which COX-2 mRNA levels had been measured (30,31). In general, polyclonal antibodies tended to have relatively weaker staining with higher levels of nonspecific staining than monoclonal antibodies (A. Ristimäki, MD, PhD, unpublished data, 2005); thus, we used a monoclonal antibody in this study. An additional challenge is that atypia lesions are small (generally <0.2 cm in diameter) and focal, thereby rendering our assay vulnerable to sampling artifacts. Hence, the clinical utility of immunohistochemical assessment of COX-2 will require additional research to establish assay consistency and reproducibility. Nevertheless, we were able to study a sizable cohort of women with atypical hyperplasia who had long follow-up time for the subsequent development of breast cancer. The association between COX-2 status and outcome, as well as the observed dose–effect relationship between development of breast cancer and the level of COX-2 staining in atypias, indicates that COX-2 may have utility as a predictive biomarker. Findings in this study support the association between NSAID treatment and risk reduction in breast cancer and also the potential to devise individualized chemoprevention approaches based on patient-specific biomarker assays. For example, in a recent study, Chan et al. (32) showed that efficacy of aspirin as a chemoprevention strategy for colorectal carcinoma was limited to patients with tumors that express COX-2.

In summary, we found moderate to strong COX-2 expression in 44% of atypical hyperplasia samples from a well-characterized patient cohort. Samples with three or more foci of atypia, which are associated with increased risk of subsequent breast cancer (2), had stronger COX-2 staining. However, the relationship between COX-2 staining intensity and the risk of subsequent breast cancer was of only borderline statistical significance ($P$ = .07).

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